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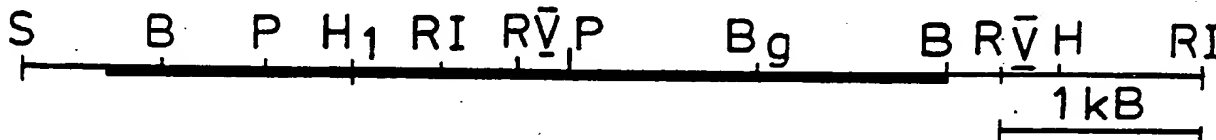


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## (57) Abstract

Yeasts are disclosed which incorporate a DNA sequence which is such that, when sequence is expressed in the yeast, the yeast does not require osmotic buffering for growth. When the expression product of the DNA sequence is no longer available within the yeast, the latter will lyse. The sequence may be incorporated in the chromosomal DNA of the yeast host or on a plasmid incorporated in the yeast. Also disclosed is a disruptant mutant wherein in the mutant the coding sequence of the gene which confers osmotic stability is interrupted whereby the mutant may be grown in a non-osmotically buffered medium but becomes osmotically sensitive upon transfer to an osmotically buffered medium.

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## SRB1 ENCODING GENE AND YEASTS TRANSFORMED WITH THIS GENE

The present invention relates to yeasts.

The yeast Saccharomyces cerevisiae is the best characterised eukaryote in terms of its genetics and molecular biology and is a very useful vehicle organism for gene cloning studies(1). One particularly useful property of this organism is its ability to form large multi-subunit protein complexes. This ability has been exploited in the production of vaccines such as that for Hepatitis B where the subunit protein, synthesised in yeast, assembles into a large particle reminiscent of that found in the natural host of the virus(2). This results in the presentation of the viral antigen in an authentic and highly immunogenic form. S. cerevisiae contains a transposon (called Ty) which has a number of features in common with mammalian retroviruses(3), and which produces a virus-like particle which is retained within the host yeast cell(4). It has been suggested(5) that Ty particles might be useful carriers for a number of viral antigens including that of the AIDS virus (HIV). It may well be that antigens presented in this way will prove to be highly effective vaccines. For these applications it is obviously impossible to excrete a large particle from the cell and therefore the particles must be recovered from harvested yeast.

The first step in the recovery of a protein or particulate product retained within the cell must be cell breakage. Yeast has a particularly tough cell wall and its breakage by mechanical means (sonication or pressure changes) requires considerable power inputs. An alternative approach is to digest the wall with lytic enzymes, but this adds to the cost of the process and can result in damage to the product by contaminating proteases. What is required is a yeast cell which will spontaneously lyse to release the product.

A number of lysis mutants has been isolated but little is known about how the genes defined by these mutations determine the ability of the yeast cell wall and/or membrane to withstand such stresses as elevated temperature and osmotic shock.

Temperature-sensitive cly mutants which lyse after prolonged incubation at the restrictive temperature of 36°C were isolated by

Hartwell (6, 7), but nothing is known about their mechanism of lysis. Mutants lacking most of the outer chain of the N-linked mannoproteins of the cell wall have been reported (8). These mnn9 mutants exhibit a physically distorted cell wall, grow slowly, and gradually lyse during their growth. A third class of yeast cell lysis mutants is defined by the fragile mutant isolated by Venkov et al(9). Cells of this mutant grow normally in media containing osmotic stabilisers (10% sorbitol or mannitol; 1.6% NaCl) but lyse spontaneously on transfer to a hypotonic solution. The efficiency of lysis is directly proportional to the change in osmotic pressure between the growth and lysis media, i.e. the greater the concentration of osmoticum in the growth medium the greater the degree of lysis on transfer of the cells to aqueous media (10).

The original fragile mutant, VY1160 (9) carried at least three mutations one of which determines lysis ability and the other two, temperature-sensitivity(11). The mutation determining osmotic fragility and lysis ability has been outcrossed; it defines a single chromosomal gene in which the fragile, mutant, allele (srbl-1) is recessive to the non-fragile, wild-type, allele (SRB1) (11). Moreover the phenotypic effect of the srbl-1 mutation is suppressed by strains carrying ochre-suppressing tRNAs and thus must represent a chain-terminating mutation within a protein-encoding gene.

Saccharomyces cerevisiae (our strain designation 7SLU) carrying the srbl-1 mutation has been deposited in the National Collection of Yeast Cultures (Colney Lane, Norwich, Great Britain) under Accession No. 2344 and has the characteristic of requiring osmotic buffering (eg. 10% w/v sorbitol) for growth but of lysing on subsequent transfer to hypotonic medium. The deposit was made on 13th July 1990 under the provisions of the Budapest Treaty on the International Recognition of the deposit of microorganisms for the purpose of patent procedure.

For convenience in the following description, reference will be made to the term "srbl mutation" which is intended to be generic to the srbl-1 mutation itself as well as mutations, variations, and derivatives at the SRB1 locus which have the same or similar phenotypic effects. The designation srbl-1 is intended specifically to refer to the mutant allele isolated by Venkov (9).

The present invention has been based on the cloning of the SRB1

gene with the object of providing DNA sequences which complement the srbl mutation in a yeast host.

Therefore, according to a first aspect of the present invention there is provided an isolated DNA sequence which complements the srbl mutation of a yeast host such that when this DNA sequence is used to transform the host and is expressed therein the host does not require osmotic buffering for growth. For convenience the DNA sequence of the second aspect of the invention is referred to herein as the "complementing sequence".

One complementing sequence in accordance with the first aspect of the invention is the DNA insert (which we designate as SRB1) in the YEpl3 plasmid (LEU2 2u origin, pBR 322, 4.8Kb insert containing SRB1) deposited in the National Collection of Yeast Cultures (Colney Lane, Norwich, Great Britain) under Accession No. 2343. The deposit was made on under the provisions of the Budapest Treaty on the International Recognition of the deposit of microorganisms for the purpose of patent procedure. This sequence provides a second aspect of the present invention, as do fragments (particularly the BamHI fragment) and allelic variations of this sequence which are capable of complementing the srbl mutation.

A particular yeast host which may be transformed by the complementing sequence of the second or third aspect of the invention is Saccharomyces cerevisiae 7SLU.

Whilst we do not wish to be bound by any particular theory, we believe that the complementing sequences of the first and second aspects of the invention (when used to transform a host carrying the srbl mutation (eg. Saccharomyces cerevisiae 7SLU) expresses a protein which is effective to prevent lysis of the cells upon osmotic shock. Mutants which do not carry a functional Srb1 protein will lyse upon transfer from an osmotically buffered medium to a hypotonic environment (ie.osmotic shock). The dependence on the presence of an osmotic buffer for growth displayed by strains carrying the srbl-1 mutant allele is probably the result of the production of a fragment of the Srb1 protein.

The provision of these complementing sequences therefore renders possible the transformation of yeast strains carrying this

mutation such that the transformant does not require osmotic buffering for growth (during which a useful internalised product may be produced) but nevertheless the cells may lyse to release the internalised product when a certain condition is satisfied.

Therefore, according to a third aspect of the present invention there is provided a yeast carrying the srbl mutation which has been transformed so as to include a copy of the DNA sequence of the second (or third) aspect of the invention which is capable of being expressed within the cells such that the cells are capable of growth in a medium which is not osmotically buffered, and said cells lysing when the expressed product of the DNA sequence is no longer available within the cell.

Such a transformed yeast host has utility for the production of a desired product which is not excreted from the yeast cells. In this case, the gene for producing the desired product is incorporated in the host, and the host cultivated by standard techniques. Upon lysis of the yeast, the desired product is released for subsequent collection/isolation.

In accordance with the third aspect of the present invention, the DNA sequence which complements the srbl mutation may be provided in the yeast chromosome. In this case, various mechanisms are available for ensuring that the expressed product is no longer available within the cells, and specific examples are detailed more fully below.

As an alternative to incorporation of the complementing sequence in the chromosomal DNA of the yeast strain, the complementing sequence may be provided in a cloning vector (particularly a plasmid) which may be used to transform the yeast.

Therefore, according to a fourth aspect of the present invention there is provided a cloning vector comprising a complementing sequence in accordance with the first or second aspect of the invention under the control of its own or another promoter inserted in a plasmid which replicates within a yeast carrying the srbl mutation. In this embodiment, the expression product preventing lysis is produced within the cell only so long as the cell incorporates plasmid(s) including the complementing sequence. Loss of all of these plasmids from the cell will result in lysis thereof.

As thus far described, the invention has related particularly to lysis of yeast cells carrying the srbl mutation.

A fifth aspect of the present invention relates to a further development in relation to the lysis of yeasts and according to this fifth aspect there is provided a genetically engineered disruptant mutant of a yeast strain wherein in the mutant the coding sequence of the gene which confers osmotic stability (ie. a DNA sequence corresponding to that of the first or second aspect of the invention) is interrupted whereby the mutant may be grown in non-osmotically buffered medium but becomes osmotically sensitive upon transfer to an osmotically buffered medium.

This fifth aspect of the invention has resulted from our studies of the SRB1 gene which will be described with specific reference to Saccharomyces Cerevisiae YPH274, although is applicable to other wild type yeasts.

Saccharomyces cerevisiae YPH274 (16) is a diploid strain which carries two wild type copies of the SRB1 gene one on each of a pair of homologous chromosomes and is therefore osmotically independent. It would therefore be assumed that disruption of the coding sequence of this gene would result in a mutant strain having a similar phenotype to that conferred by srbl-1. We have however established that this is not the case and that a distinct phenotype is produced. More particularly, the phenotype of the disruptant mutant permits its growth in a medium which does not contain an osmotic buffer but allows lysis ability to be induced on transfer to an osmotically buffered medium, the cells being caused to lyse when re-transferred to a non-osmotically buffered medium.

An example of the way in which such a disruptant mutant may be produced is described below.

The invention will be further described by way of example only with reference to the accompanying drawings, in which:

Fig. 1 is a restriction map of the original cloned fragment which complements srbl-1 and which contains the SRB1 gene;

Fig. 2 is a restriction map of a YEpSS1 plasmid;

Figs. 3 and 4 are graphs illustrating loss of plasmids from yeast;

Fig. 5 illustrates the manner in which a plasmid vector may be constructed for producing a disruptant mutant in accordance with the fifth aspect of the invention;

Fig. 6 illustrates production of the disruptant mutant.

Referring firstly to Fig. 1, there is shown therein a restriction map of a DNA fragment containing the SRB1 gene which is capable of complementing the srb1-1 mutation. The DNA fragment is incorporated in the YEpl3::SRB1 plasmid is deposited under Accession No 2343 as identified above. In fig. 1 the thin lines represent the plasmid vector DNA. The darker lines represent genomic DNA. Restriction sites: RI-EcoRI; RV-EcoRV; B-BamHI; Bg-BglIII; P-PstI; S-SalI; H-HindIII; Hl-HpaI. The procedure by which we have cloned the SRB1 gene is outlined in Appendix A which also gives further characterising information relating to the gene.

In one embodiment of the invention the cloned segment of yeast genomic DNA containing the SRB1 gene (or the BamHI fragment thereof) is integrated into the chromosomal DNA of a srb1-1 Saccharomyces cerevisiae such as 7SLU and placed under the control of a regulatable yeast promoter such as that from the GAL1,10 gene (18). Alternatively a similar construct could be incorporated into a self-replicative plasmid. The SRB1 gene will thus be expressed when galactose is present in the growth medium, but not in its absence. The Saccharomyces cerevisiae 7SLU may also be genetically engineered to incorporate a gene (which may be provided on a plasmid) which expresses a desired product which is retained within the host cell. This product may for example be a protein or a virus-like particle, eg. Human superoxide dismutase, Bovine chymosin, or Hepatitis B surface antigen.

The transformed strain may then be grown in a medium (comprising for example 1% yeast extract, 2% peptone, and 2% galactose) without osmotic buffering which includes inter alia galactose. The presence of galactose in the medium ensures that the SRB1 gene is expressed so that the cells grow normally and there is no lysis. Throughout this growth period, the desired product is produced and remains within the cell. However, when galactose is exhausted, there is no expression of the SRB1 gene with the result that the product of

this expression is no longer present in the cells which therefore spontaneously lyse. The amount of galactose provided in the growth medium may be calculated such that it is growth-limiting and the cells will cease to grow and then lyse on exhaustion of galactose. Once cell lysis has taken place, the originally internalised desired product is released into the bulk medium and may be recovered by standard techniques.

In a modification of the above procedure, the Saccharomyces cerevisiae 7SLU may incorporate a gene sequence (eg. a synthetic sequence) which may be transcribed, from a regulatable promoter, into an anti-sense RNA to the SRB1 gene or transcript. In this case, the cells may be grown in standard media and at some appropriate time during this growth the appropriate substrate is added to the growth medium to induce transcription of the anti-sense gene. As a result, expression of the SRB1 gene is switched off so that the expression product is no longer available within the cell which therefore lyses as previously.

A further embodiment of the invention relates to a self-selecting plasmid system. The stability of multicopy plasmids in cultures of genetically engineered microorganisms, including yeast, is a significant barrier to the efficient production of recombinant proteins (20, 21). A self-selecting plasmid system is one in which cells which have lost the plasmid cease to grow, even in poorly-defined media. In accordance with this embodiment of the invention, the DNA fragment containing the SRB1 gene may be inserted into a suitable replicative plasmid and will be expressed from either its own or some other promoter which is functional in yeast. This SRB1 containing vector may then be used to transform Saccharomyces cerevisiae 7SLU, although it should be noted that the vector molecule used should be one which is capable of replicating as multiple copies within the host cell since otherwise the SRB1 insert is not capable of complementing the srbl-1 mutation. The vector may also incorporate a gene which expresses a desired product, eg. a protein or a particle, which remains within the cell.

The transformants containing multiple copies of the plasmid carrying the SRB1 gene are capable of growing in medium without an osmotic stabiliser so as to produce the internalised product. However

loss from the cell of the plasmids carrying the SRB1 gene (as will probably occur at some stage during growth of the cell) obviously means that this gene is no longer expressed within the cell which will therefore spontaneously lyse.

This has the advantage that non-productive cells do not accumulate in the culture and their biomass will be made available to the growing, productive yeast cells which have retained the plasmid.

To demonstrate the efficiency of this system, we have constructed a plasmid, YEpSS1 (Fig.2). This plasmid contains the 5.0 kb fragment bearing the yeast SRB1 gene cloned into the HindIII/SalI sites of plasmid YEpS1 which contains the yeast gene SOD1 encoding Mn-Superoxide dismutase (22); (23) as a model gene for a product of potential industrial importance.

The YEpSS1 plasmid was then used to transform a wild-type SRB1 host (strain DL<sup>-</sup>), as was the plasmid YEpS1. Batch culture experiments were then conducted using these transformed hosts in a medium which was not osmotically stabilised and the results are shown in Fig. 3 which is a plot of the %ge of cells containing the plasmid vs No. of Generations of Growth. It will be seen that the YEpSS1 was very unstable in the wild type (SRB1) host, with the plasmid being completely lost from the population after about 42 generations of growth (cf results obtained for plasmid YEpS1).

The plasmid YEpSS1 was then used to transform a host (SLU"15") bearing the srbl-1 mutation and batch culture experiments were conducted in which the host was grown in the presence of an osmotic stabiliser (10% sorbitol) and also in the absence of such a stabiliser. The results are shown in Table 1 below. It will be seen from the Table that for the transformed host grown in the presence of osmotic stabiliser the plasmid was completely lost from the population after 45 generations of growth. In contrast, the YEpSS1 plasmid was retained in almost 100% of the cells after 45 generations of growth in the absence of an osmotic stabiliser.

Table 1

% Plasmid - free cells			
Generations	YEpSS1		
		SLU "15" (+10% sorb.)	SLU "15"
8		61	0
16		72	0
24		84	0
30		87	0
38		95	0
46		100	0

Continuous culture experiments were conducted to confirm the above results. In this case, the YEpSS1 plasmid was used to transform yeast strain SLU"15" which was then grown under the stringent conditions of continuous culture. The results are shown in Fig.4. It will be seen that the YEpSS1 plasmid was lost from the culture after 30 generations of growth in the presence of an osmotic stabiliser (10% sorbitol) but was maintained in nearly 100% of the srb1-1 cells after 100 generations of growth in the absence of a stabiliser.

The above results show that the plasmid carrying the SRB1 gene is stably maintained in the yeast host under conditions in which the host is grown without osmotic stabiliser and therefore is consequent selection against plasmid free cells.

The foregoing description has concentrated on transformed yeasts in accordance with the third aspect of the invention and plasmid vectors in accordance with the fourth aspect. The subsequent description relates to a disruptant mutant in accordance with the fifth aspect of the invention.

Fig. 5 illustrates the construction of a vector for forming the disruptant mutant. In Fig. 5, the "open" boxes represent vector sequences, horizontal bars represent SRB1 derived sequences, and vertical bars represent TRP1 sequences. Plasmid Lp2 (Fig. 5) was

constructed as follows: The plasmid pUC18\* was produced by eliminating the EcoRI site from the polylinker of pUC18(14). This plasmid was digested with PstI and the cut ends dephosphorylated. The 1.5kb PstI fragment of SRB1 (see Fig. 1) was purified and ligated into the PstI site of pUC18\*. The PstI fragment was chosen because it includes at least part of the coding sequence of the gene and contains an EcoRI site which is within the open reading frame. It also incorporates a HpaI site which may be used until homologous integration with a corresponding site for homologous integration with the corresponding site in the SRB1 gene (see *infra*). The resultant plasmid, Lp1, was digested with EcoRI, dephosphorylated and ligated to the 1.4kb EcoRI fragment of YRp7(15) which contains the S. cerevisiae TRP1 gene. The plasmid thus obtained was designated Lp2; in it, the PstI fragment of the SRB1 gene is interrupted by the insertion of the TRP1 gene. Lp2 was used to transform the diploid yeast strain YPH274(16), which has the following genotype:

MATa ura3-52 lys2-801 (amber) ade2-101 (ochre)

MATa ura3-52 lys2-801 (amber) ade2-101 (ochre)

trp1 1 his3- 200 leu2- 1 SRB1

trp1 1 his3- 200 leu2- 1 SRB1

Lp2 was linearised by digestion with HpaI (see Fig. 6) in order to target the integration event to sequences homologous to the SRB1 clone. As a result, the linearised plasmid Lp2 was incorporated within the SRB1 gene of YPH274, thus interrupting the coding region thereof. Trp<sup>+</sup> transformants were selected and one of these, YPH274-4 used for further analysis. YPH274-4 was sporulated and 40 meiotic tetrads dissected, from which 15 complete tetrads were obtained. The TRP1 marker was found to segregate 2:2 and all haploid Trp<sup>+</sup> spore clones examined were found to be susceptible to osmotic shock, although none required an osmotic buffer for growth. Thus the transformant has the following genotypes;

MATa ura3-52 lys2-801 (amber) ade2-101 (ochre)

MAT a ura3-52 lys2-801 (amber) ade2-101 (ochre)

trp1 1 his3- 200 leu2- 1 SRB1  
trp1 1 his3- 200 leu2- 1 srbl::TRP1

None of the Trp<sup>-</sup> segregants from the same tetrads was osmotically sensitive. A haploid containing the srbl::TRP1 disruption mutation was crossed with a srbl-1 strain of opposite mating type.

The disruptant was found unable to complement the srbl-1 mutation and, moreover, haploid meiotic segregants obtained from this diploid are all sensitive to osmotic shock. These data confirm that the fragment cloned is identical to the SRB1 gene. Southern analysis with pulsed field gels(17), using the SRB1 clone as a probe, as well as appropriate control probes to identify genes of known location, has demonstrated that the SRB1 gene is located on chromosome XV.

A haploid segregant described above in which the TRP1 gene is inserted into the chromosomal copy of SRB1, thus interrupting its coding sequence and preventing its functional expression, has a number of characteristics which may be exploited in industrial processes. The mutant is not dependent on the incorporation of an osmotic stabiliser into the medium in order to grow. Thus large-scale cultures of the mutant may be grown in media which are commonly used in industrial processes. Cells from such cultures may then be transferred into an high osmotic pressure medium where they will rapidly acquire the ability to lyse on subsequent transfer to hypotonic conditions. This regime should form the basis of efficient industrial processes for the production of yeast extracts for food or feed and also for the production and recovery of recombinant proteins.

The srbl::TRP1 disruption mutant (or any srbl- mutant produced by recombinant DNA techniques and relying on the availability of the cloned SRB1 gene) may be used as a host for the stable maintenance of recombinant plasmids which bear the wild-type SRB1 gene.

This is exemplified by the following data. An srbl::TRP1 mutant strain designated 4d was transformed with the YEpSS1 plasmid (see supra) and was grown in YEPD medium with and without an osmotic stabiliser (10% sorbitol). Table 2 below shows the percentage of plasmid-free cells obtained after the indicated number of generations

of growth. It will be seen that 100% of the plasmids are retained in the cells after 48 generations of growth, both in the presence and absence of osmotic stabiliser.

For comparison, Table 2 also includes the data from Table 1 in relation to the SLU"15" strain (which loses 100% of plasmids after 48 generations of growth) and data for the wild-type strain DL<sup>-</sup> grown in the absence of an osmotic stabiliser. As shown in Table 2, 100% of the DL<sup>-</sup> cells lose the plasmid after about 40 generations of growth in a non-osmotically buffered medium.

To ensure lysis of the srbl:TRP1 mutant, it would be possible (for example) for the SRB1 gene in the plasmid incorporatd in the mutant to be under the control of a GAL promoter. Upon exhaustion of galactose from the cultivation medium and subsequent growth of the mutant in an osmotically buffered medium, the cells may be lysed by transfer to a hypotonic medium.

Table 2

% Plasmid-free cells					
Generations	yEpSS1				
	DL	SLU"15" (+ 10% sorb)	SLU"15"	4d (+ 10% sorb)	4d
8	40	61	0	0	0
16	68	72	0	0	0
24	89	84	0	0	0
32	95	87	0	0	0
40	100	95	0	0	0
48	-	100	0	0	0

The srbl::TRP1 disruption mutant (or related mutants, as described above) may be used as a host strain which is very readily transformed with DNA. Such a hypertransformable host will find application, for example, in the construction of gene banks from higher organisms. The disruption mutant exhibits particularly efficient transformation with high molecular weight plasmid molecules and should prove useful for gene bank construction using high capacity cloning vectors such as YACs (24). The low efficiency of transformation with YAC clones containing large inserts leads to a high probability of the few competent host cells in the population receiving two or more recombinant YACs and of chimaeric molecules being formed by subsequent host-mediated recombination. This problem of chimaera formation is a major barrier to the exploitation of YAC clone banks (see "Genome Analysis in the EC", Commission of the European Communities Directorate General for Science, Research and Development, 1991). The srbl::TRP1 disruption mutant gives high frequency transformation of DNA into intact cells without the need to treat with alkali metal ions.

This is exemplified by the data presented in Table 3 which shows the number of transformants per  $\mu$ g DNA.

Table 3

Strain	Genotype	Transforming DNA/M.W.in kb			
		YEp352 (5.2kb)	YEp352::Glucosamy (7.5kb)	YEp351::Amy (9kb)	YEp13::SRB1 (15kb)
4d	<u>srb1:: TRP1</u>	3600	1704	1904	YEp50::SRB1 (12kb)
4b	<u>SRB1</u>	360	37	36	680
				8	15

The srbl::TRP1 disruption mutant (or related mutants as described above) may be used as a test object in screening programmes for novel pharmaceutical agents for use in human or animal health or agricultural chemicals (e.g. herbicides and pesticides). Genetically engineered yeast strains have been shown to be favourable objects for such screens (25) but the cell wall presents a significant barrier to the entry of such agents into the yeast. The original srbl-1 mutant is significantly more sensitive to the action of antibiotics and bioactive peptides than wild-type cells (26), (27), (28) and we have demonstrated that the srbl::TRP1 disruptant also exhibits hypersensitivity to drugs (29). The disruption mutant is a much more favourable tool for such screening programmes than the original srbl-1 mutant since it may be grown on the surface of agar plates in the absence of an osmotic stabiliser.

The srbl::TRP1 disruptant (or related mutants as described above) may be used as a host strain which permits the enhanced secretion of proteins encoded by either yeast genes or by heterologous genes introduced into the mutant using recombinant DNA techniques.

APPENDIX A

A DNA fragment which complemented the srbl-1 mutation was isolated from a YEpl3-based yeast genomic library (12). The yeast genomic library used for the cloning of the SRB1 gene is based on the vector YEpl3(12). It is a S. cerevisiae/E. coli shuttle vector; 10.8kb in size, which contains the LEU2 gene as a marker for selection and the 2 $\mu$  DNA origin for replication in yeast; and pBR322 sequences for selection and replication in E. coli.

The genomic bank was constructed by ligating Sau3A partially digested S. cerevisiae strain S288C genomic DNA (average size 5kb fragments) into the BamHI site of the YEpl3 vector. This ligation mixture was then transformed into E. coli RR1. The transformants were pooled together and used for the isolation of recombinant plasmid DNA.

S. cerevisiae 1SL (MATa srbl leu2 tsl) was transformed with this pool of DNA and a transformant was isolated which was able to grow without sorbitol. This transformant was also tested for its lysis ability in comparison to the parental untransformed strain and was found incapable of lysis upon osmotic shock.

Total yeast DNA was isolated from this putative SRB1-containing transformant and used to transform E. coli HB101. Several E. coli transformants were tested and found to contain a 15.5kb recombinant plasmid. This plasmid, designated YEpl3::SRB1 (as deposited under NYCC Accession No. 2343, was used to transform other srbl mutants and, in all cases, was able to complement both their growth dependence upon the addition of 10% sorbitol to the nutritional medium and their ability for spontaneous lysis upon osmotic shock.

The YEpl3::SRB1 clone contained a 4.8kb insert. This insert was subjected to restriction analysis and a physical map of the fragment is presented in Fig. 1. The following restriction enzymes were found to have no site in the SRB1 fragment: XmaI, BclI, PvuII, BstEII, NruI, XbaI, XhoI, BglI, ClaI, SacI, KpnI. Sub-cloning experiments were carried out and it was demonstrated that it was not possible to complement the srbl-1 mutation with any fragment smaller than the 4.0kb BamHI fragment. Moreover, this fragment was only capable of

complementation when present on a vector molecule which replicates as multiple copies within the host yeast cell. A combination of restriction, Southern and Northern analyses permitted the following additional conclusions drawn about the SRB1 clone:

- i) The EcoRI site (Fig. 1) is internal to the gene and this site may therefore be used for gene disruption studies.
- ii) The 1.5kb PstI fragment is internal to the gene.
- iii) The gene encodes a 2.3kb polyadenylated mRNA species.

The YEp13::SRB1 clone and subclones constructed from it were tested for the ability to suppress well-characterised amber and ochre alleles in appropriate tester strains. The SRB1 clone was found to be unable to complement these suppressible alleles and, therefore, it is concluded that the cloned fragment probably contains the gene of interest rather than a suppressor gene.

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N.B. We now designate the original mutant allele isolated by Venkov, srbl-1, to distinguish it from our disruptant and any subsequent srbl mutants isolated.
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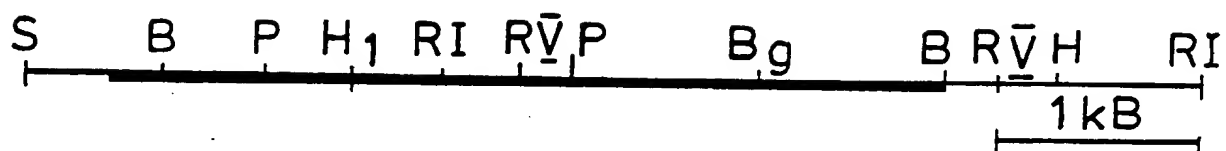
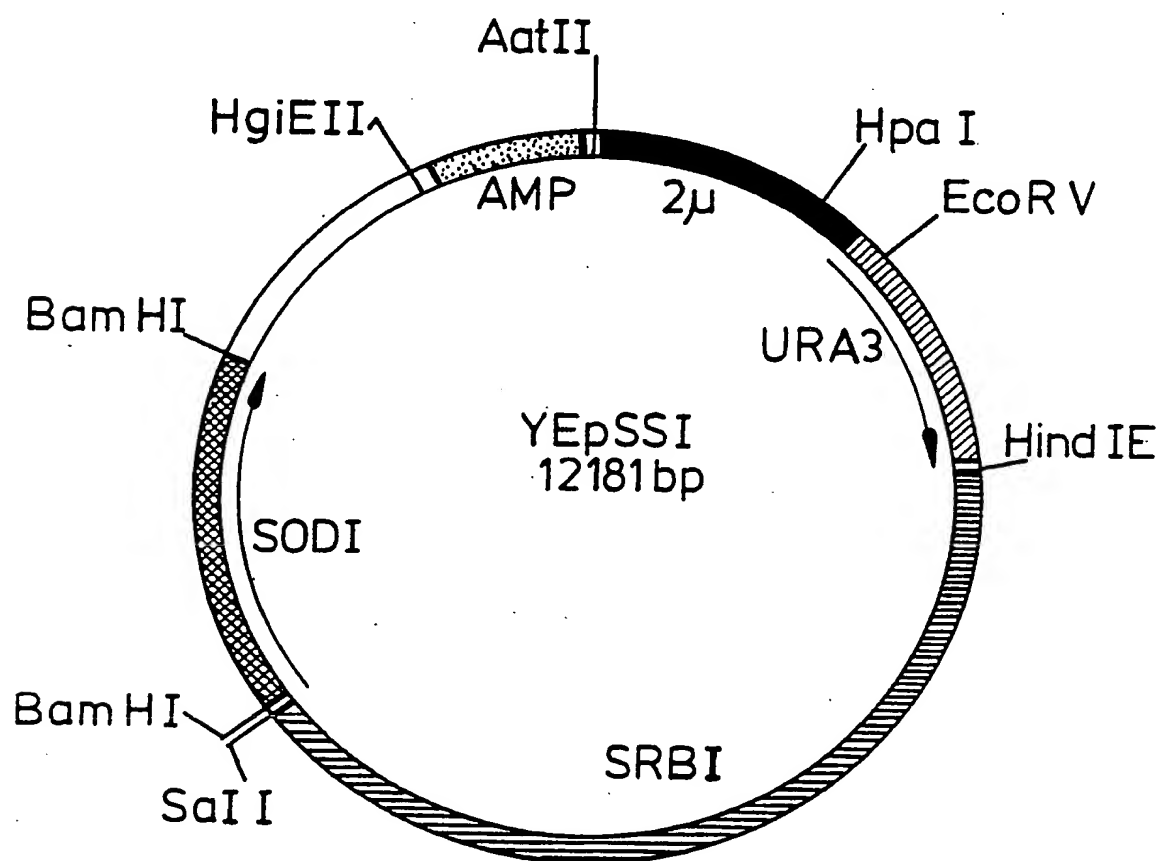
CLAIMS

1. An isolated DNA sequence which complements the srbl mutation of a yeast host carrying such a mutation such that when this DNA sequence is used to transform the host and is expressed therein the host does not require osmotic buffering for growth.
2. A DNA sequence which is the DNA insert in the YEpl3 plasmid deposited under Accession No. NCYC 2343.
3. A DNA sequence which is a fragment of the insert defined in claim 2 and which complements the srbl mutation of a yeast host carrying such a mutation.
4. A DNA sequence which is the BamHI fragment of the DNA insert in the YEpl3 plasmid deposited under Accession No. NCYC 2343.
5. A DNA sequence which is an allelic variation of a sequence as defined in any one of claims 1 to 4.
6. A cloning vector for transforming a yeast host carrying the srbl mutation, the vector comprising a DNA sequence as claimed in any one of claims 1 to 5 under the control of its own or another promoter inserted in a plasmid which replicates within the yeast host.
7. A cloning vector as claimed in claim 6 wherein the plasmid is capable of replicating as multiple copies within the yeast host.
8. A cloning vector as claimed in claim 6 or 7 further carrying a gene for expressing a desired product which is not excreted from the yeast cell.
9. A transformed yeast host comprising the yeast carrying the srbl mutation which has been transformed with a DNA sequence as claimed in any one of claims 1 to 4 or a cloning vector as claimed in any one of claims 5 to 8.
10. A transformed host as claimed in claim 9 wherein said DNA sequence is incorporated in the chromosomal DNA of the host.
11. A transformed host as claimed in claim 9 wherein said DNA sequence is carried on a plasmid.
12. A transformed yeast host as claimed in any one of claims 9 to 11 wherein the yeast carrying the srbl mutation is that deposited under Accession No. NCYC 2344.
13. A method of obtaining a desired product by cultivating a yeast, the product being one which is not excreted from the yeast, the

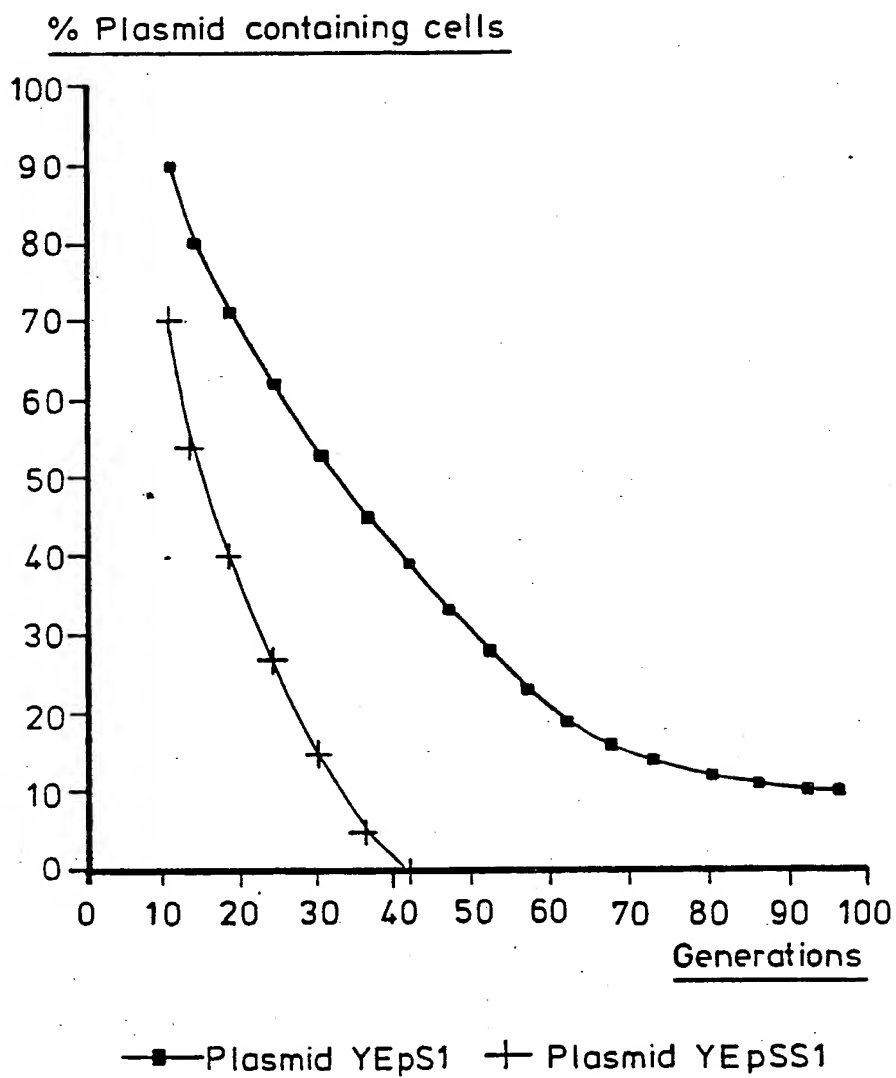
method comprising

- (i) providing a yeast host which carries the srb1 mutation and which further incorporates a DNA sequence complementing the srb1 mutation together with a gene for expressing the desired product,
  - (ii) cultivating the yeast host in a non-osmotically buffered medium so as to express both the gene and the DNA sequence,
  - (iii) allowing the cells to lyse when the expression product of said DNA sequence is no longer available therein, and
  - (iv) recovering the desired product.
14. A genetically engineered srb1 mutant of a yeast strain.
  15. A genetically engineered disruptant mutant of a yeast strain wherein in the mutant the coding sequence of the gene which confers osmotic stability is interrupted whereby the mutant may be grown in a non-osmotically buffered medium but becomes osmotically sensitive upon transfer to an osmotically buffered medium.
  16. A disruptant mutant as claimed in claim 15 wherein the gene whereof the coding sequence is interrupted is the SRB1 gene.
  17. A mutant as claimed in claim 16 wherein the coding sequence of the SRB1 gene is disrupted by homologous recombination into the chromosomal copy of the gene by a cloned fragment of the SRB1 gene.
  18. A mutant as claimed in claim 17 wherein the integration is at the HpaI site of the gene by means of a gene fragment incorporating the corresponding HpaI site.
  19. A mutant as claimed in claim 18 wherein the gene fragment is PstI fragment.
  20. A mutant as claimed in claim 19 wherein the PstI fragment is interrupted by a marker.
  21. A mutant as claimed in claim 20 wherein the marker is the TRP1 gene.
  22. A mutant as claimed in any one of claims 12 to 19 which incorporates a plasmid carrying the SRB1 gene.

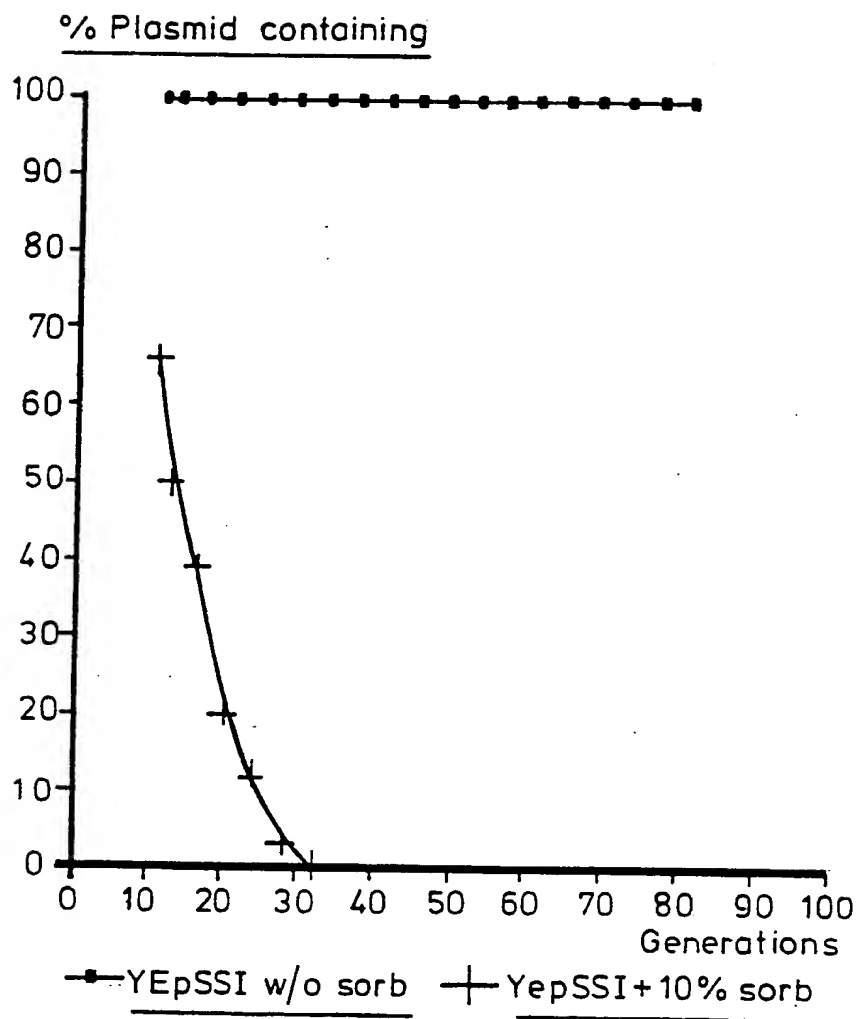
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FIG. 1FIG. 2

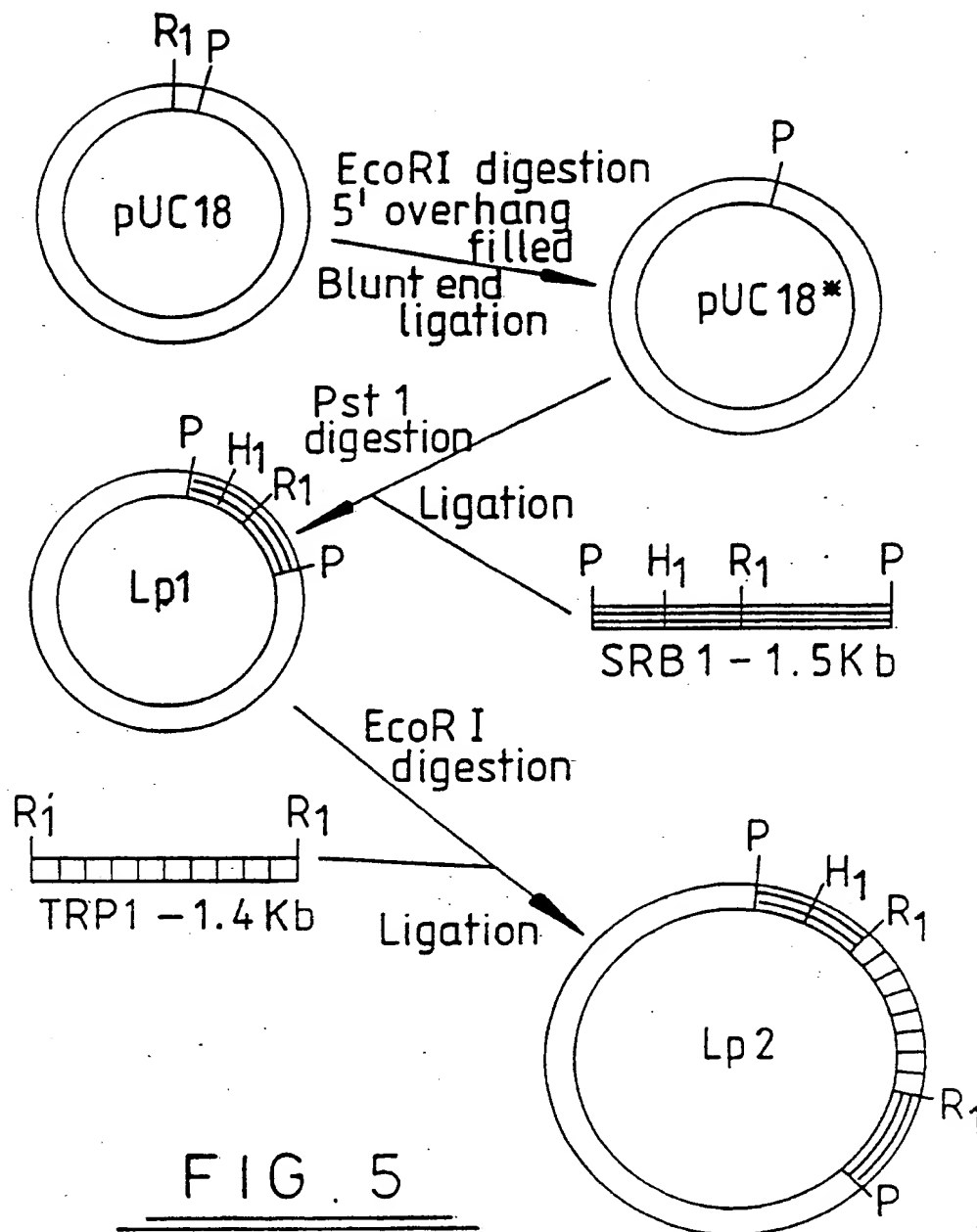
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FIG. 3

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FIG. 4

4 / 5

FIG. 5

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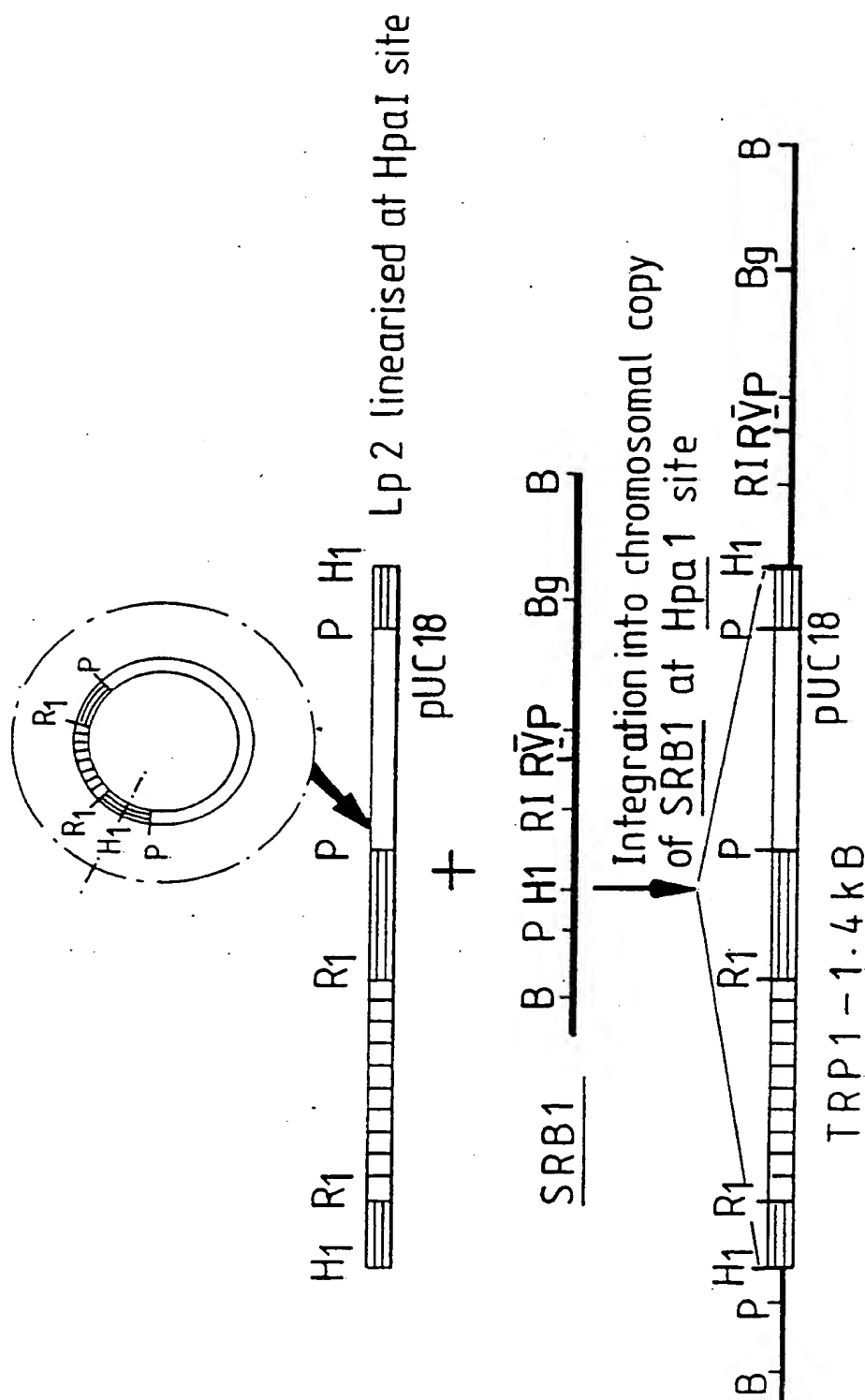


FIG. 6

**MICROORGANISMS**Optional Sheet in connection with the microorganism referred to on page 3, line 12-14 of the description \***A. IDENTIFICATION OF DEPOSIT \***Further deposits are identified on an additional sheet ☒ \*

Name of depositary institution \*

National Collection of Yeast Cultures

Address of depositary institution (including postal code and country) \*

Colney Lane,  
Northwich,  
Great Britain.

Date of deposit \*

13th July 1990

Accession Number \*

NCYC 2343

**B. ADDITIONAL INDICATIONS \*** (leave blank if not applicable). This information is continued on a separate attached sheet ☐**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE \*** (if the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS \*** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later \* (Specify the general nature of the indications e.g. "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

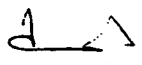
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau is

was

18 NOVEMBER 1991  
(18. 11. 91)

(Authorized Officer)

  
J. L. Baum

## INTERNATIONAL SEARCH REPORT

International Application

PCT/GB 91/01224

I. CLASSIFICATION SUBJECT MATTER (If several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/81; C07K15/00; C12N1/19; C12P21/02

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System

Classification Symbols

Int.Cl. 5

C12N ; C07K

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	YEAST vol. 4, 1988, CHICESTER, UK pages 219 - 225; L.I. STATEVA, P.V. VENKOV, A.A. HADJIOLOV, L.A. KOLEVA AND N.L. LYUDSKAHOV: 'Polyploid fragile strains of <i>Saccharomyces cerevisiae</i> - a novel source of proteins for nutritional purposes' cited in the application ---	
P, X	MOLECULAR AND CELLULAR BIOLOGY vol. 11, no. 8, August 1991, WASHINGTON, DC, USA pages 4235 - 4243; L.I. STATEVA, S.G. OLIVER, L.J. TRUEMAN AND P.V. VENKOV: 'Cloning and characterization of a gene which determines osmotic stability in <i>Saccharomyces cerevisiae</i> ' --- -/-	1-22

<sup>10</sup> Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

04 NOVEMBER 1991

Date of Mailing of this International Search Report

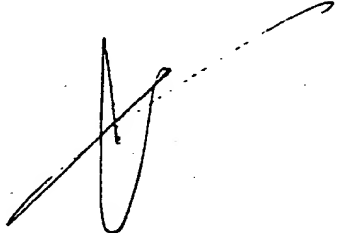
19. 11. 91

International Searching Authority

EUR PEAN PATENT OFFICE

Signature of Authorized Officer

VAN PUTTEN A.J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	
P,X	EP,A,0 411 896 (MERCK & CO., INC.) 6 February 1991  	15

GB 9101224  
SA 50300

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